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Identification of Zinc Finger Proteins Bound to a Silencer Region in the Rat Glutathione Transferase P Gene

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The rat glutathione transferase P (GST-P) gene is strongly induced during chemical hepatocarcinogenesis, whereas mRNA of this gene is rarely expressed in normal rat liver. We previously identified a silencer region in the promoter of this gene. This silencer has several DNA binding sites and at least three proteins (Silencer factor-A, -B, and -C (SF-A, SF-B, and SF-C)) bind to these sites. We previously cloned and characterized the Nuclear Factor 1 (NF1) family and the CCAAT/enhancer-binding protein (C/EBP) family as SF-A and SF-B, respectively. However, SF-C which binds to GST-P silencer 2 (GPS2) remains to be cloned. By screening using yeast one-hybrid system, several zinc finger proteins were identified as a candidate of SF-C. The gel-mobility shift analyses showed that BTEB2, EZF, LKLF, TFIIIA, TIEG1, and novel zinc finger protein MZFP bound to GPS2 with different affinities. Several proteins of these are known to be transcriptional activators or repressors, suggesting that zinc finger proteins bind to GPS2 and regulate GST-P expression in the rat liver.

Key words glutathione transferase P; silencer; zinc finger protein; transcription; carcinogenesis

Rat glutathione transferase P (GST-P) gene expression is undetectable in normal rat liver, but its expression level increases significantly during the early stage of chemical hepatocarcinogenesis. Therefore, GST-P is known to be an excellent tumor marker.^{1–4} Previous studies identified the enhancer and silencer regions upstream of the GST-P gene.^{5–7} The enhancer activates the gene expression, while the silencer represses it. The GST-P silencer exists between –396 and –140 bp on the GST-P gene promoter and consists of several DNA binding sites (Fig. 1).⁸ At least three proteins bind to these *cis*-elements, the GST-P silencer (GPS), and are named Silencer factor-A, -B, and -C (SF-A, SF-B, and SF-C). We already reported that SF-A is a Nuclear Factor 1 (NF1) family and SF-B is a CCAAT/enhancer-binding protein (C/EBP) family.^{9,10}

The NF1 family has four independent genes; NF1-A, -B, -C, and -X.^{11–13} In these family members, we identified NF1-A as an SF-A which bound to several sites (GPS0A, GPS0B, GPS3, GPS4, GPS5 and site 7) in the GST-P silencer region.^{8,9} We also reported that NF1-A has four splicing isoforms NF1-A1–4, and all of them have repression ac-

tivities. Two repression domains were mapped in the carboxyl half in the NF1-A, and the identified repression domains were rich in serine and glycine, and proline and serine, respectively.^{14,15}

The C/EBP family was identified as an SF-B which bound to GPS1.¹⁰ This family consists of six genes: C/EBP α , β , γ , δ , ϵ , and C/EBP homologous protein 10 (CHOP10).¹⁷ Western blot analyses of the C/EBP isoforms during chemical hepatocarcinogenesis revealed a decrease in C/EBP α expression. However, there was no change in the C/EBP β level. In the nuclear extracts from normal liver, C/EBP α was the dominant form that bound to GPS1, whereas both C/EBP α and C/EBP β bound to GPS1 in the nuclear extracts from carcinogenic liver. Furthermore, the transfection analyses showed that C/EBP α not only repressed the GST-P promoter activity but also attenuated the transcriptional stimulation by C/EBP β . These observations strongly suggest that the ratio of C/EBP α to C/EBP β is one of the important factors for the GST-P silencer activity, and the decrease in this ratio during hepatocarcinogenesis reduces the silencer activity, and consequently increases the GST-P expression.¹⁷

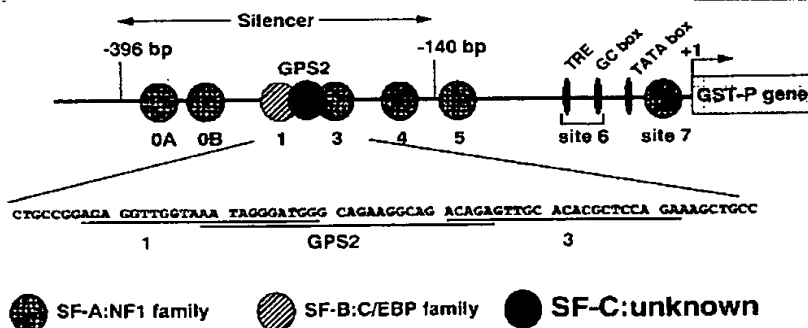


Fig. 1. Schematic Diagram of the Promoter and Silencer Regions in the Rat GST-P Gene

SF-C binding site as well as SF-A and SF-B are shown. SF-C binding site GPS2 partially overlaps with GPS1 and GPS3, which are bound by the C/EBP family and the NF1 family, respectively.

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In this way, we identified two transcription factor families, NF1 and C/EBP as silencer binding proteins in the GST-P gene. However, the SF-C which binds to GPS2 remains to be characterized. According to the transfection assay using deletion mutants in the rat fibroblast cell line 3Y1, GPS2 works as a down-regulation site, and the footprint analysis showed the existence of the heat labile protein bound to GPS2.⁸⁾ First, we tried to purify SF-C using a combination of chromatographic techniques or to isolate SF-C cDNA by the Southwestern technique.⁸⁻¹⁰⁾ However, we could not obtain GPS2 binding protein, SF-C.

In the present study, we performed the yeast one-hybrid assay using GPS2 as a binding sequence, and cloned eight zinc finger proteins from the rat liver, HeLa, Jurkat, and WI-38 cDNA libraries. In these factors, BTEB2 (basic transcription element binding protein 2), EZF (epithelial zinc finger), LKLF (lung Krüppel-like factor), TFIIIA (transcription factor IIIA), TIEG1 (TGF β -inducible early transcription factor gene 1), and novel zinc finger protein MZFP (multiple zinc finger protein) appeared to be GPS2 binding factors.

MATERIALS AND METHODS

Yeast One-Hybrid System and cDNA Libraries Yeast MATCHMAKER one-hybrid system and MATCHMAKER GAL4 cDNA libraries of rat liver, HeLa, Jurkat, and WI-38 were obtained from Clontech Laboratories, Inc., and used briefly according to the manufacturer's instructions.

Preparation of Yeast Reporter Strain Two oligonucleotides for the DNA sequence spanning GPS2 of the GST-P gene was synthesized with *Bam*HI sites at the 5' and 3' ends as follows:

5'-gatcAATAGGGATGGGCAGAAGGCAG
TTATCCCTACCCGTCTTCCGTCctag-3'

As a negative control *cis*-element, the DNA sequences spanning ERT, which is an enhancer element in the GST-P gene, was synthesized with *Xba*I sites at the 5'-and 3' ends as follows:

5'-ctagAGTAGTCAGTCACTA
TCATCAGTCAGTGATgatc-3'

These double strand oligonucleotides were multimerized to a 5-mer (GPS2) or 4-mer (ERT) and subcloned into the *Bam*HI site (GPS2) or *Xba*I site (ERT) of pUC18. A fragment containing 5xGPS2 was ligated into pHISi (5xGPS2-pHISi) and pLacZi (5xGPS2-pLacZi), and a fragment containing 4xERT was ligated into pHISi-1 (4xERT-pHISi-1), and pLacZi (4xERT-pLacZi) (these three plasmids having *ura3* gene as a selection marker were from Clontech Laboratories, Inc.). The 5xGPS2-pLacZi digested at *Xho*I and 5xGPS2-pHISi digested at *Nco*I were transformed into the yeast strain YM4271 (MATa, *ura3-52*, *his3-200*, *ade2-101*, *lys2-891*, *leu2-3*, *112*, *trp1-903*, *tyr1-501*) (termed YM4271/GPS2), and the 4xERT-pLacZi digested at *Xho*I and 4xERT-pHISi-1 digested at *Nco*I were also transformed into YM4271 (termed YM4271/ERT) by the lithium acetate method¹⁸⁾ with slight modification as described below.

Transformation of Reporter Plasmids into Yeast YM4271 was grown to OD₆₀₀ of 0.6 in 200 ml YPD (1% yeast extract, 2% tryptone, 2% glucose). The cells were har-

vested and suspended in 1 ml lithium sorbitol (100 mM lithium acetate, 10 mM Tris-HCl pH8.0, 1 mM EDTA, 1 M sorbitol). One hundred micrograms of salmon sperm DNA and 1 μ g of linearized reporter plasmids were mixed with 50 μ l of cell suspension and 300 μ l of 40% polyethylene glycol in lithium sorbitol. After incubation at 30°C for 30 min, the mixture was added to 45 μ l of dimethyl sulfoxide (DMSO) and incubated at 42°C for 15 min. The selection of the transformant with the *lacZ* reporter was performed on synthesis dropout (SD) plates lacking uracil, while the leaky HIS3 expression from pHISi and pHISi-1 was used for *his3* reporter integration, and transformants were selected on SD plates lacking histidine.

Yeast One-Hybrid Screening After determining the background level of the expression of YM4271/GPS2, the plasmids of the cDNA libraries, which have *leu2* gene as a selection marker, from rat liver, HeLa, Jurkat, and WI-38 were transformed into YM4271/GPS2 by the lithium acetate method at a 20-fold larger scale than the transformation for the preparation of the reporter strain. Then, the positive clones were selected on SD plates lacking leucine and histidine but containing 10 mM 3-aminotriazol (3-AT) (first screening). The positive clones were streaked on papers (Whatmann #5) on SD plates lacking leucine and histidine but containing 10 mM 3-AT and the growing colonies were further selected by β -galactosidase assay (second screening). Next, the plasmids from the positive clones were prepared by disruption using glass beads and transformed into YM4271/GPS2. The re-transformed independent colonies were tested for β -galactosidase assay (third screening). Then, the plasmids were prepared from the positive clones and transformed into *E. coli* strain DH-5 α by the electroporation method. Plasmid DNA was isolated and used for the fourth screening and sequencing.

Finally, the plasmids were transformed into YM4271/GPS2 and YM4271/ERT, and then the growth on the SD plates lacking leucine and histidine but containing 10 mM 3-AT was observed, and β -galactosidase assay was performed (fourth screening). The nucleotide sequence of the resultant positive clones were determined by the dye terminator method (ABI 310 sequencer).

Production of Protein in Bacteria *Eco*RI and *Xho*I fragments containing cDNA of the positive clones were removed and the 5' and 3' ends were filled in by Klenow enzyme (Toyobo Inc.). The fragments were ligated at the *Sma*I site of pGEX-2T (Amersham Pharmacia Biotech) and transformed into *E. coli* strain JM109. The plasmids prepared from the transformant were sequenced by the dye terminator method (ABI 310 sequencer) and transformed into *E. coli* strain BL21. The transformants were grown overnight at 30°C in Luria-Bertani (LB) medium containing 100 μ g/ml ampicillin, inoculated into 100 ml LB medium, and grown to OD₆₀₀ of 0.6, at which time isopropyl- β -D-thio-galactopyranoside (IPTG) was added to achieve a final concentration of 0.4 mM. The cells were then allowed to grow for an additional 4 h at 30°C, harvested by centrifugation and suspended in 0.1 M HM buffer (25 mM HEPES-KOH (pH 7.8), 1 mM dithiothreitol, 12.5 mM MgCl₂, 20% glycerol, 0.1 M KCl) containing 1 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml pepstatinA, and 0.3 μ g/ml antipain and disrupted by sonication. Following centrifugation at 350000 $\times g$ for 30 min, the GST-fusion pro-

tein in the supernatant was purified by Glutathione Sepharose 4B (Amersham Pharmacia Biotech) and the size of GST-fusion protein was checked by SDS-polyacrylamide gel electrophoresis. Then, these were used for gel-mobility shift analyses.

Gel-Mobility Shift Analyses A gel-mobility shift analyses was performed as previously described¹⁹⁾ with a slight modification. In brief, 6.25 μ l of the protein fraction was mixed with same volume of the reaction buffer (20 mM Tris-HCl (pH 7.5), 10% glycerol, 2 mM dithiothreitol, 10 mM EDTA, 0.5 μ g of poly (dl-dC), 12.5 μ g of bovine serum albumin, and 0.2 ng of labeled probe) as well as the competitors. The binding reaction was performed at room temperature for 30 min. The oligonucleotide sequences of both the probes and competitors are indicated as follows:

GPS2: 5'-ctagAATAGGGATGGGCAGAAGGCAG-3'
3'-TTATCCCTACCGTCTTCCGTCgac-5'

GC box: 5'-agctTCCGTTGGGGCGGGGCTTCACG-3'
3'-AGGCAACCCCGCCCGAAGTGCagct-5'

GT box: 5'-agctTCCGTTGGGGTGTGGCTTCACG-3'
3'-AGGCAACCCCGACCCGAAGTGCagct-5'

δ A: 5'-ctagGACTCAATTTCCCAATGTAGCT-3'
3'-CTGAGTTAAAGGGTTACATCGagac-5'

RESULTS

Cloning of GPS2 Interacting Factors by Yeast One-Hybrid Screening In this study, we have cloned SF-C, which is a GPS2 binding protein in the GST-P silencer, using the yeast one-hybrid system, and screened from the rat liver cDNA library and also the human cDNA libraries of HeLa, Jurkat, and WI-38. As summarized in Table 1, four screening steps were performed. At the first screening, the effector plasmids from each library were transformed into the host strain containing 5-times repeats of GPS2 harboring *his3* and *lacZ* genes in its chromosome as a reporter (YM4271/GPS2). More than 6.0×10^6 plasmids in each library were transformed into yeast. This number was 2-fold greater than that of the independent clones from each library. The positive clones expressing the *his3* gene were selected on SD plates lacking leucine and histidine but containing 10 mM 3-AT. The number of positive clones from each library at each step are indicated in Table 1.

For the second screening, the positive clones after the first screening were streaked on the paper-sheeted SD plate lacking leucine and histidine but containing 10 mM 3-AT and then the *lacZ* reporter gene expression was detected using the β -galactosidase assay. In the β -galactosidase assay, the positive clones turned blue. By this screening, the number of positive clones was reduced down to about half (Table 1).

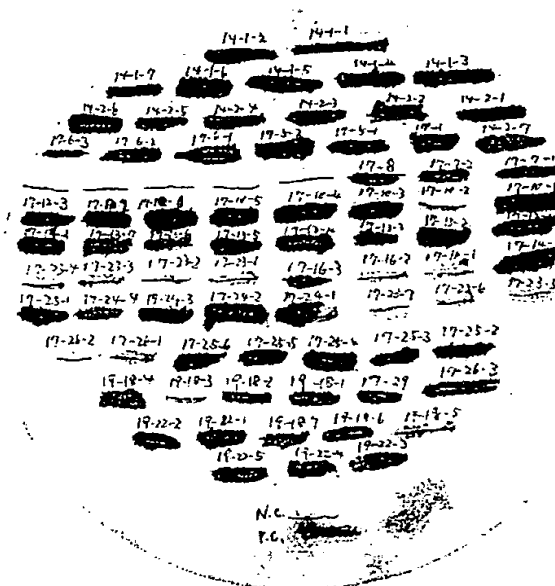
Since we suspected that the positive clones contained more than one effector plasmid per cell, we performed a third screening. At this step, the plasmids prepared from the positive clones after the second screening were again transformed into the host strain YM4271/GPS2. Then, we checked the yeast growth on SD plates and performed the β -galactosidase assay. By this selection, more than 70% of the clones were omitted as false positive clones (Table 1 and Fig. 2).

Next, the plasmids from positive clones after the third

Table 1. Summary of SF-C Screening by the Yeast One Hybrid System

Source of libraries	Rat liver	HeLa	WI-38	Jurkat
Number of screened clones	1.2×10^7	6.9×10^6	1.0×10^7	1.0×10^7
Number of positive clones in 1st screening	57	160	81	333
Number of positive clones in 2nd screening	33	101	37	131
Number of positive clones in 3rd screening	1	54	6	21
Number of positive clones in 4th screening	1	33	6	11
Identified genes	Sp4 (1)	BTEB2 (6) EZF (24) TFIIIA (1) TIEG1 (1)	TIEG1 (2)	CPBP (2) LKLF (2) MZFP (2) TFIIIA (2) TIEG1 (1)

The number of positive clones at each step for screening are shown. The numbers in parentheses at the identified genes are the number of clones isolated.



plates and turned blue in the β -galactosidase assay. In contrast, the transformed YM4271/ERT could not grow on the SD plates lacking leucine and histidine but containing 10 mM 3-AT. The transformants growing on the SD plate lacking leucine could not show the β -galactosidase activity. The typi-

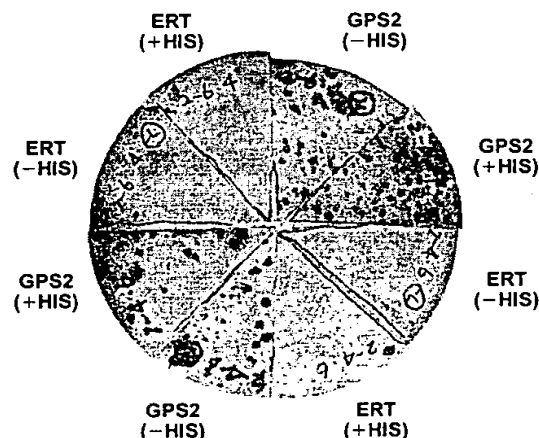


Fig. 3. β -Galactosidase Assay for the Fourth Screening

The plasmids prepared from the positive clones in the third screening were transformed into *E. coli* DH-5 α . After plasmid preparation from DH-5 α the plasmids were transformed into the yeast host strains YM4271/GPS2 and YM4271/ERT. The transformants were spread on the SD plates lacking leucine and histidine but containing 10 mM 3-AT (-His) and the SD plate lacking leucine (+His). The colonies grown on the SD plates were transferred to paper and the β -galactosidase assay was performed. The findings of screenings using the Jurkat cDNA library is shown.

cal pattern of the positive clone is shown in Fig. 3. The effector plasmids finally identified as positive clones were sequenced.

The sequence analyses of positive clones finally revealed 8 independent genes. Sp4 (Specific protein 4) was obtained from both the rat and human libraries, and BTEB2, CPBP (core promoter-binding protein), EZF, LKLF, TFIIIA, TIEG1, and a part of PAC clone RP4-751H13 (GenBank AC004877) were all from the human cDNA libraries (Table 1 and Fig. 4). This PAC clone is derived from human chromosome 7q35 with 128361 bases, and the region of 20009–21523 bases seems to code the novel zinc finger-like protein. Therefore, we named multiple zinc finger protein (MZFP) here, although its expression has not been reported and its function is not yet clear.

All genes cloned here had the C₂H₂ type zinc finger motif. In these genes, MZFP and TFIIIA had 12 and 8 zinc finger motifs, respectively, and the other 6 genes had three tandem zinc fingers, the so-called Krüppel-like zinc finger domain. In Fig. 4, these zinc finger domains are illustrated, and the region obtained by the yeast one-hybrid assay is indicated as black bars. We cloned the full length of the open reading frame (ORF) of BTEB2, but the other clones contained only a partial sequence of ORF, although all 8 clones included the entire zinc finger domain.

DNA-Binding Specificity of GPS2 Interacting Proteins
Since all of the factors cloned as GPS2 interacting proteins by the yeast one-hybrid system had the zinc finger motifs,

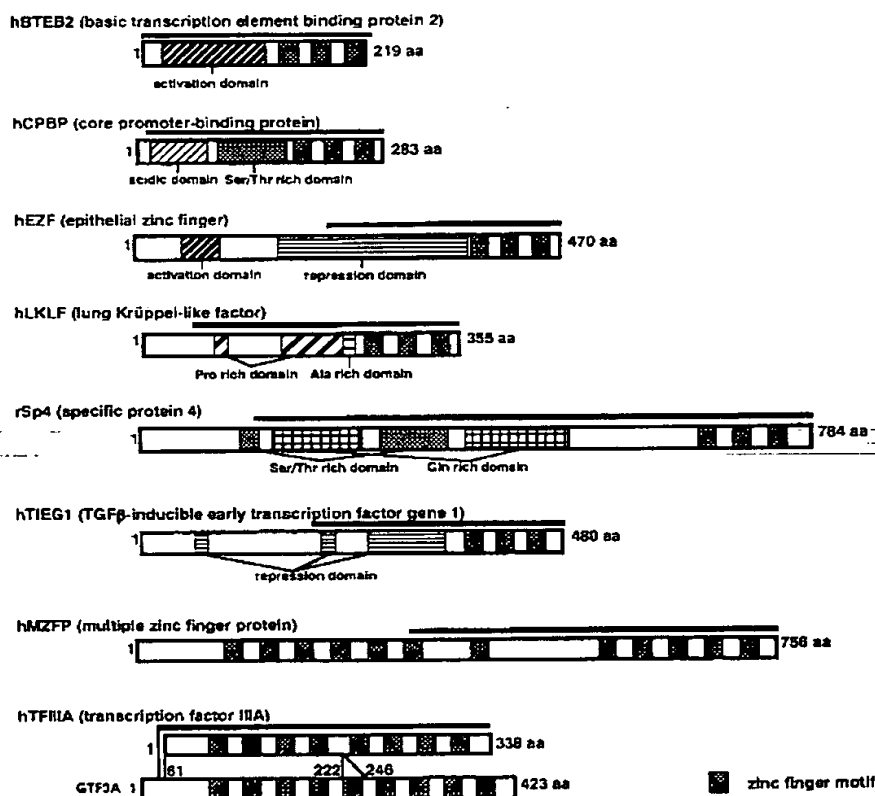


Fig. 4. Schematic Illustration of GPS2 Interacted Factors in the Yeast One-Hybrid Screening

The factors cloned by the yeast one-hybrid screening are shown. The black bars above each factor indicate the region obtained by the screening.

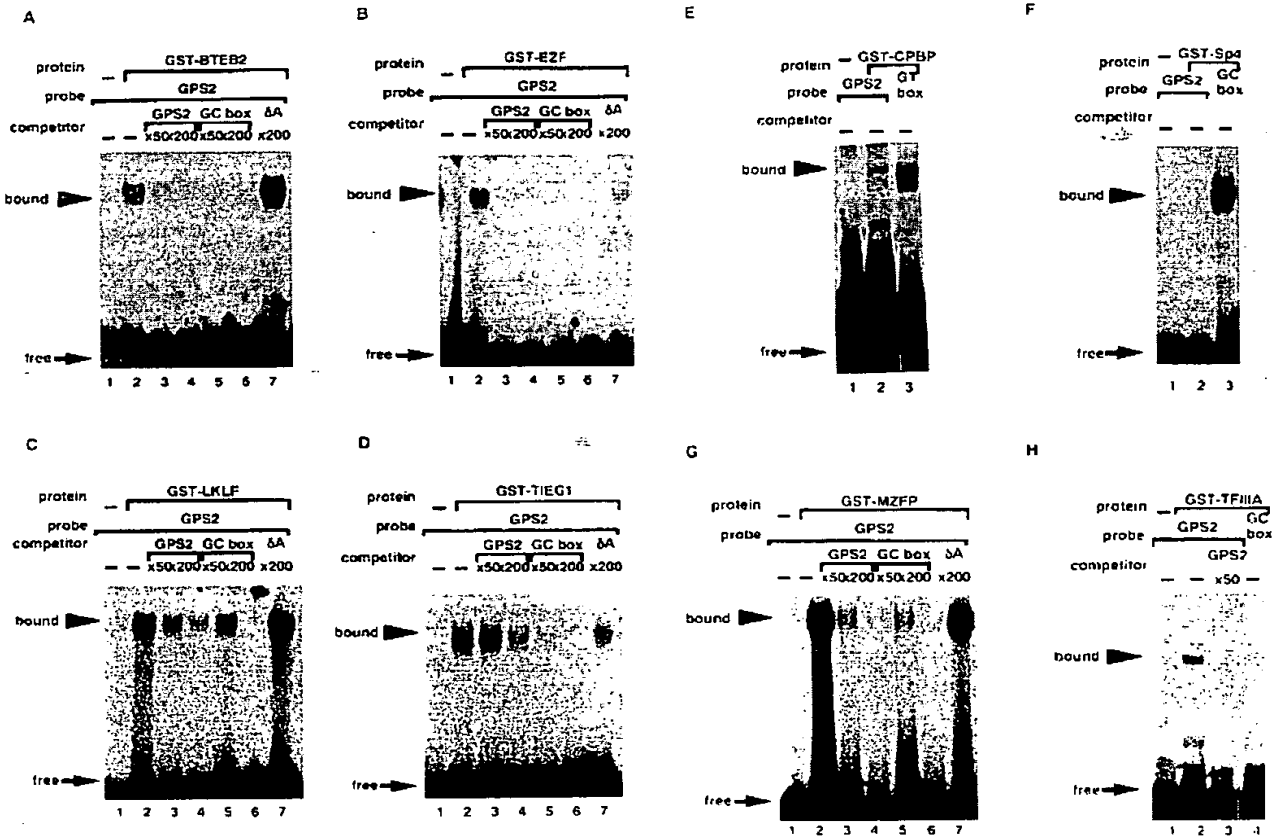


Fig. 5. Gel-Mobility Shift Assay for GPS2 Binding Proteins

The Gel-mobility shift assay was performed using bacterially expressed GST-fusion proteins and the radiolabeled GPS2 or the GT box or the GC box as indicated in each panel. Lane 1 in each panel was a control with bovine serum albumin added. The protein-bound band and protein-free band are indicated by arrowheads and arrows, respectively. Either 50- or 200-fold molar excess of non-labeled GPS2 and the GC box were used for the competition analysis. ΔA was used as a non-specific competitor. The radiolabeled GPS2 was incubated with (A) GST-BTEB2 (lanes 2–7), (B) GST-EZF (lanes 2–7), (C) GST-LKLF (lanes 2–7), and (D) GST-TIEG1 (lanes 2–7). (E) The radiolabeled GPS2 and the GT box were incubated with GST-CPBP (lanes 2, 3). (F) The radiolabeled GPS2 and the GC box were incubated with GST-Sp4 (lanes 2, 3). (G) The radiolabeled GPS2 was incubated with GST-MZFP (lanes 2–7). (H) The radiolabeled GPS2 and the GC box were incubated with GST-TFIIIA (lanes 2–4).

which is known as DNA binding domain, this suggested that these factors bind to GPS2 through the zinc finger domain. To test which factor binds to GPS2 specifically, we performed gel-mobility shift analysis using bacterially expressed GST fusion proteins. As a probe, we used GC box or the GT box in addition to GPS2, because some of these proteins are known to bind to the GC box and/or the GT box.

When the GST-BTEB2 fusion protein and GPS2 as a probe were used, the retarded band was observed (Fig. 5A lane 2). This band disappeared with the addition of a 50- or 200-fold molar excess of both the non-labeled GPS2 and GC box (Fig. 5A lanes 3–6) but not with the addition of the 200-fold molar excess of non-specific DNA ΔA , which is a C/EBP δ binding site. The GC box competitively blocked the binding between GST-BTEB2 and radiolabeled GPS2 more strongly than GPS2.

Among other Krüppel like proteins, GST-EZF, GST-LKLF, and GST-TIEG1 showed the similar findings (Figs. 5B–D). However, GST-CPBP and GST-Sp4 bound to the GT box and the GC box but did not bind to GPS2 (Figs. 5E, F). As shown in Table 1 and Fig. 4, we cloned the C-terminal half of MZFP and this region contained 6 zinc finger motives. When the gel-mobility shift assay was performed using GST-MZFP in-

cluding the C-terminal half, we observed a similar finding as observed using GST-BTEB2 (Fig. 5G). That is, GST-MZFP specifically bound to GPS2. TFIIIA was shown to bind to DNA through the N-terminal three zinc fingers.²⁰ Therefore, we amplified the region coding the N-terminal three zinc fingers by polymerase chain reaction (PCR) and expressed as the GST-fusion protein. Using this protein, GST-TFIIIA was found to be bound to GPS2. Interestingly, the affinity of TFIIIA to GPS2 was stronger than that to the GC box and the GT box (Fig. 5H lanes 2, 4, and data not shown).

DISCUSSION

Although the expression of the rat GST-P gene is undetectable in normal rat liver, it is induced when the rat is treated with a carcinogen such as acetylaminofluorene (AAF) and becomes hepatocarcinoma.^{1–4} Therefore, the GST-P gene is known as a tumor marker and it appears important to clarify how the GST-P gene expression is regulated for resolving the mechanism of hepatocarcinogenesis. In previous studies, we reported that the GST-P gene has an enhancer and a silencer upstream of this gene which regulate gene expression positively and negatively, respectively.^{5–7} It is neces-

sary to clarify how the GST-P gene expression is kept at an undetectable level in normal rat liver.

The silencer region exists between -396 bp and -140 bp of the GST-P gene.⁵⁾ At least three proteins, SF-A, SF-B and SF-C are known to bind to the silencer and these binding sites down-regulate the expression of the GST-P gene.⁸⁾ SF-A and SF-B were identified as an NF1 family and a C/EBP family, respectively.⁸⁻¹⁰⁾ However, the GPS2 binding protein, SF-C has not yet been identified, although GPS2 was found to be a *cis*-element repressing the GST-P gene expression in transfection analysis.⁸⁾

We previously cloned SF-A and SF-B using a combination of chromatographic techniques and the Southwestern method, respectively.⁸⁻¹⁰⁾ We were unable to clone SF-C using similar techniques. In the present study we cloned SF-C using the yeast one-hybrid system. Using this technique, we obtained eight genes as GPS2 interacting proteins; BTEB2, CPBP, EZF, LKLF, Sp4, TFIIIA, TIEG1, and the novel zinc finger protein MZFP. All had C₂H₂ type zinc finger motives. As shown in Fig. 4, these proteins were divided into two groups; proteins containing three tandem repeat C₂H₂ type zinc fingers, so-called Krüppel like zinc finger domain, and proteins containing multiple zinc finger motives.

The sequence of human TFIIIA was reported by two studies, and the isoforms may be produced by splicing.^{21,22)} Drew *et al.*, reported a partial sequence of human TFIIIA and Arakawa *et al.*, reported the whole sequence of ORF as GTF3A. The former reported that TFIIIA lacked 60 amino acids at the N-terminus of GTF3A, whereas the fifth zinc finger motif (222-246 amino acid) of GTF3A was spliced out in human TFIIIA. The sequence of C-terminus of the present clone is identical to that reported by Drew *et al.* However, the additional 18 bp of present clone at 5'-side coincides with GTF3A. Therefore, in Fig. 4, we showed the both structures of TFIIIA and GTF3A with our clone.

The yeast one-hybrid system was unable to indicate the direct interaction between GPS2 and these proteins. To determine whether these proteins bind to GPS2 directly, we performed gel-mobility shift analyses. These assays revealed that, among Krüppel like proteins, BTEB2, EZF, LKLF and TIEG1 bound to GPS2, while CPBP and Sp4 could bind weakly and could not bind to GPS2, respectively. The bindings of former four proteins to GPS2 were competitively blocked by the non-labeled GPS2 and GC box. These findings indicate that BTEB2, EZF, LKLF and TIEG1 bind to GPS2 as well as GC box.

The binding manner of the multiple zinc finger protein, MZFP, was similar to that of Krüppel like zinc finger proteins described above. However, TFIIIA binds to GPS2 relatively stronger than the GC box, because the binding of TFIIIA to GC box was rarely observed. Taken together, a total of 6 proteins were identified as candidates for GPS2 binding proteins, although there were some differences in binding affinities.

Human BTEB2 was reported as a transcriptional activator bound to the GC box.^{23,24)} The transfection analysis using GAL4 fusion protein suggested that human EZF has both activation and repression domains. According to the transfection analysis using full length human EZF and reporter plasmid containing EZF binding sites, EZF repressed the tk-promoter activity.²⁵⁾ However, mouse EZF activated the tran-

scriptional activity.²⁶⁾ The mouse LKLF was reported as an activator.²⁷⁾ Human TIEG1 represses the transcriptional activity.²⁸⁾ Thus, these Krüppel like proteins identified in the present study were known to be a transcriptional activator and/or repressor.

TFIIIA is the prototype of the zinc finger superfamily.²⁹⁾ TFIIIA binding to the internal control region (ICR) of 5S rRNA gene, recruits TFIIIB and TFIIIC and promotes transcription of RNA polymerase III.³⁰⁻³²⁾ It has not yet been reported that TFIIIA binds to regulation region of class II genes and regulate their expression. This possibility remains to be resolved. Moreover, TFIIIA binds to GPS2 more tightly than to the GC box and the GT box compared with other zinc finger proteins isolated in the present study.

In the present study, we isolated 6 genes as candidates for GPS2 binding proteins, and it is not clear which one(s) are the real factor regulating the silencer function through GPS2. The expression profiles of these proteins except MZFP in normal liver of various animals were reported. The expression levels of BTEB2 in rat liver, EZF in mouse liver, and LKLF in rat and human liver are very low.^{23,26,27)} GTF3A (TFIIIA) expresses at a high level in human liver.²²⁾ While TIEG1 highly expresses in mouse liver, it expresses at a low level in rat and human liver.³³⁻³⁵⁾ Therefore, the expression pattern of these proteins in both normal and hepatoma livers, and also in hyperplastic nodules during hepatocarcinogenesis must be characterized, and the detailed analyses of the transfection are also required. Furthermore, since GPS2 site overlaps with GPS1 and GPS3, to which C/EBP family and NF1 family bind (Fig. 1), the functional interactions among these proteins including the competition to binding sites and interaction with other cofactors remain to be resolved.

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